Hmox1 (Heme Oxygenase-1) Protects Against Ischemia-Mediated Injury via Stabilization of HIF-1α (Hypoxia-Inducible Factor-1α)

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Published in:
Arteriosclerosis, Thrombosis, and Vascular Biology

DOI:
10.1161/ATVBAHA.120.315393

Publication date:
2021

Document Version
Peer reviewed version

SUPPLEMENTAL MATERIAL

Detailed Methods

Murine hind limb ischemia

Unilateral hind limb ischemia was surgically introduced in ~12-week-old male and female *Hmox1*$^{-/-}$ mice and sex-matched *Hmox1*$^{+/+}$ or *Hmox1*$^{+/-}$ littermates. Mice were anesthetized with isoflurane (IsoFlo®, Abbott Laboratories) (4% induction, 2% maintenance, 0.8 L/min) and administered carprofen analgesia pre-operatively (5 mg/kg s.c.; Tergive®, Parnell) before undergoing surgery on a heated mat. A skin incision was made over the left femoral artery, the artery and vein isolated between the profunda and epigastric arterial branches and the popliteal bifurcation, and 6-0 Prolene (Ethicon) sutures placed proximal to the branches and bifurcation. The femoral artery and vein were resected between the ligatures to create a deep tissue ischemia. The right femoral artery was subjected to a sham preparation as described for the left femoral artery without ligation and resection of the femoral artery and vein. For both the left and right femoral arteries, the incisions were closed and the animal allowed to fully recover ambulant movement before being returned to group housing.

Bone marrow transplantation

Male 6-12 weeks-old *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ donor mice were used for bone marrow isolation. The femurs and tibias of donor mice were flushed with RPMI media. The cell suspension was mixed, filtered through 100 μm nylon mesh then centrifuged at 930*g* for 5 min. The supernatant was aspirated, and the cell pellet re-suspended in RPMI at a final concentration of 5×10^7 cells/mL. At 6 weeks-of- age, female recipient *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ mice were subjected to lethal irradiation (6.5 Gy). Following irradiation, the drinking water was supplemented with amoxicillin (0.35 mg/mL). On day 1 following irradiation, 1×10^7 bone marrow cells from either male *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ mice were administered in a 200 μL volume by tail vein injection using a 25 G needle. Female mice underwent hind limb ischemia surgery after a 6-week period of bone marrow engraftment.

Laser Doppler perfusion imaging

All scans were performed at ambient room temperature (21-25 °C) with the mouse on a heating pad (37 °C). To minimize variations in temperature a minimum of two consecutive scans were performed. In brief, deeply anesthetized mice were positioned supine with their hind limbs externally rotated immediately prior to (baseline, Pre) and following hind limb surgery (day 0), and again on days 1, 3, 7, 10, 14, 17 and 21. Doppler images were assessed using moorLDI Software Version 5.2 (Moor Instruments). Using the ankle as an anatomical reference point, regions of interest were created over the paw area and the total blood perfusion calculated. Blood perfusion was assigned a 0 value if amputations occurred above the ankle. Blood perfusion results were expressed as a ratio of the ischemic hind limb to the non-ischemic hind limb.

Histological analyses

Gastrocnemii were removed and snap-frozen in liquid nitrogen. Tissues were orientated in O.C.T^{TM} Compound (Tissue-Tek) and cryosections (5 μm) prepared as follows: sectioning commenced immediately above the distal tendon and proceeded proximally, with serial sections collected every 200 μm for a total distance of 1,000 μm. Sections were allowed to adhere to SuperFrost Plus glass slides (Lomb). For vessel density studies sections were fixed in ice-cold acetone for 10 min at 4 °C. Sections were washed 4 times in phosphate-buffered saline (PBS) then blocked for 30 min at room temperature in PBS containing 0.25% Triton

X-100 and 1% bovine serum albumin (BSA) (PBST). Next, slides were incubated with antibodies (or isotype controls) in PBST for 60 min at room temperature; rat anti-CD31-phycoerythrin (CD31-PE clone 390; 1:100, Abcam, ab25644), mouse anti-smooth muscle actin-fluorescein isothiocyanate (SMA-FITC; 1:500, Sigma-Aldrich, F3777), rabbit anti-laminin (1:1000, Abcam, ab11575). Slides were washed 4 times in PBS then incubated for 20 min at room temperature in goat anti-rabbit Alexa Fluor 350 (1:2000, Thermo Fisher Scientific, A- 11046). Slides were washed 4 times in PBST then cover-slipped in ProLong Gold Antifade (Thermo Fisher Scientific). Fluorescent photomicrographs (3 fields per 5 sections per mouse) were taken on the Zeiss Axio Imager M1 using the 20x objective and Zen Blue Edition 2012 Software (scale bar = 100 μm). Lumen diameter, and number of dual CD31-PE, SMA-FITC vessels and myocytes were enumerated using ImageJ software by observers blinded to genotype and treatment. SMA-FITC^{+} vessels were then classified by diameter (<50 μm, 50-100 μm, >100 μm) according to American Heart Association Guidelines[1].

An independent veterinary pathologist (Rothwell Consulting, Sydney, Australia) blinded to genotype, treatment and mouse strain assessed hind limb tissue and cell morphology by hematoxylin and eosin staining. Apoptosis was next assessed in serial sections by two methods. TUNEL staining was performed on gastrocnemius sections in accordance with the supplied protocol (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, Merck-Millipore, S7100). DNAse I-treated sections were used as a positive control. For active caspase-3 staining, slides were fixed and blocked as described above then incubated with rabbit-anti active caspase-3 antibody (1:100, Abcam, ab49822) for 2 h at room temperature. Slides were washed 4 times in PBST, incubated for 30 min in biotinylated anti-rabbit antibody (1:1000, DAKO, E0466), washed 4 times in PBST then incubated in streptavidin HRP antibody (1:1000, DAKO, P0937). Following a final 4 washes slides were developed with DAB chromagen (DAKO, K3467). Both TUNEL and active caspase-3 sections were counter-stained in 0.1% methylene blue.

Fibroblast isolation and treatments

Fibroblast lines were individually established from male *Hmox1*$^{+/+}$ (n=4) and *Hmox1*$^{-/-}$ (n=4) mice using a skin explant technique[2] with minor modifications. In brief, hair was removed, and the dorsum washed in 70% ethanol before dissection from the subcutaneous tissue. The dorsum was incubated in trypsin (0.25%)-EDTA (0.05%) for 60 min at 37 °C then the epidermis and remaining subcutaneous tissue removed with a scalpel. Ten 3 × 3 mm squares of skin were placed per well of fibronectin- coated 6-well plates (BD Biosciences) and a sterile coverslip placed on top. Next, 2 mL DMEM containing 20% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin was added. Cells were cultured under standard tissue culture conditions (95% air, 5% CO_2 at 37 °C, humidified).

***In vitro* glucose uptake assay**

Fibroblasts were plated in a 12-well culture plate at 1×10^5 cells per well in 2 mL DMEM + 10% FBS under standard tissue culture conditions. The following day fibroblasts were transferred to the hypoxic chamber (1% O_2, 5% CO_2 at 37 °C humidified) or kept under standard tissue culture conditions. Cells were incubated for 8 h then gently washed with warm PBS. This was followed by the addition of 1 mL 200 nM insulin in PBS + 1 g/L BSA, and the plates were further incubated for 30 min at 37 °C. Insulin solution was removed and cells gently washed twice in PBS before addition of 1 mL glucose solution (final concentration 0.02 μCi/mL [^{3}H]-2-deoxyglucose, 100 μM 2-deoxy- D-glucose ± 20 μM cytochalasin B (to account for non-glucose transporter-mediated uptake) in warm PBS + 1

g/L BSA). Cells were incubated for 5 min, washed gently 3 times with ice-cold PBS then lysed in 1% SDS. Next, 400 µL digest were added to 4 mL scintillant (Ultima-Flo, Perkin Elmer), vortexed and then counted for radioactivity (LS6500 Liquid Scintillation Counter, Beckman Coulter). Protein concentration of lysate was determined by the bicinchoninic acid assay (Pierce, Thermo Fisher Scientific).

***Ex vivo* glucose uptake assay**

Skeletal muscle glucose uptake was performed as previously described[3]. In brief, soleus muscle was carefully removed from the hind limb of mice, bisected longitudinally into two strips then pre- incubated for 30 min in Krebs Henseleit buffer (5.5 mM glucose, 2 mM pyruvate and 1 g/L BSA, pH 7.3). Pre-incubation and subsequent incubations were all performed at 30 °C with continuous gassing of the buffers with carbogen (95% O_2, 5% CO_2). Soleus strips were transferred to fresh buffer containing 0.375 µCi/mL of [^{3}H]-2-deoxyglucose and 0.05 µCi/mL of [^{14}C]-1-D-mannitol for a period of 20 min. After incubations, muscles were rinsed in ice-cold PBS, quickly blotted on filter paper then snap-frozen in liquid nitrogen. Tendons were removed and the muscle weighed before digestion in 500 µL 1 M KOH for 20 min at 70 °C, with vortexing. Next, 400 µL digest was added to 4 mL scintillant (Ultima-Flo AF, Perkin Elmer), vortexed and then counted for radioactivity (LS6500, Beckman Coulter) with glucose uptake determined from the intracellular accumulation of [^{3}H]-2-deoxyglucose.

High resolution respirometry

High resolution respirometry was conducted as described previously[4, 5]. Approximately 2 mg skeletal muscle fibers were prepared by mechanical separation of fiber bundles using fine-tip angled forceps in BIOPS buffer (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM ATP, 6.56 mM $MgCl_2$, 20 mM taurine, 15 mM phosphocreatine, 20 mM imidazole, 0.5 mM DTT, 50 mM MES, pH 7.1) on an ice slurry. Fibers were permeabilized with saponin (50 µg/mL) in BIOPS for 30 min at 4 °C with gentle agitation. Fibers were then washed in assay buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA, pH 7.1) for 10 min at 4 °C with gentle agitation. The wet weight of the skeletal muscle fibers was recorded prior to respiration analysis.

The substrate-uncoupler-inhibition titration (SUIT) protocol was conducted to analyze skeletal muscle respiration[5]. Briefly, fibers were equilibrated for 30 min prior to sequential addition of the following substrates (final concentrations stated): 5 mM malate, 0.2 mM octanoylcarnitine, 0.63 mM adenosine diphosphate (ADP), 10 mM glutamate, 10 mM succinate and a final addition of 1.88 mM ADP. Mitochondrial electron transport system was then uncoupled using a titration of 0.5 µM, 1 µM and 1.25 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone and inhibited by the sequential addition of 0.5 µM rotenone and 2.5 µM antimycin A.

Measurement of skeletal muscle biliverdin

Approximately 15 mg of plantaris was pulverized in a liquid nitrogen bath then homogenized 2 × 30 s on ice in 5 volumes of phosphate buffer pH 7.4 containing 100 µM desferrioxamine (DFO), 100 µM diethylenetriaminepentaacetic acid (DTPA) and protease inhibitors (cOmplete™ Protease Inhibitor Cocktail, Roche) using a Heidolph homogenizer set at 7,000 rpm. Samples were centrifuged at 400*g* for 5 min at 4 °C and protein concentration of the supernatant determined by BCA assay. To 50 µL supernatant, 5 µL 0.5 µM mesobiliverdin (internal standard) were added and the solution extracted in 195 µL ice-cold methanol containing 100 µM DFO and 100 µM DTPA by mixing vigorously for 30 s. The extracted

solution was then incubated on ice for 5 min, centrifuged at 13,000*g* for 10 min at 4 °C before the resulting supernatant was subjected to LC-MS/MS analysis for the determination of biliverdin, as described previously[6].

Protein isolation and Western blot analysis

Mixed fiber-type gastrocnemius tissue was pulverized in a liquid nitrogen bath. Fibroblasts were washed with ice-cold PBS then placed on dry ice. SDS-urea buffer was used to prepare homogenates for Hif1α assessment. Lysates were centrifuged at 16,000*g* for 20 min at 4 °C. Proteins were separated by SDS-PAGE using 3-8% Tris-Acetate or 10% Bis-Tris gels (Thermo Fisher Scientific) and transferred to nitrocellulose membranes using the iBlot2TM (Thermo Fisher Scientific). Blots were stained with Ponceau S, imaged for total protein loading then washed in Tris-buffered saline with 0.1% Tween 20 (TBST). Blots were incubated with primary antibody at 4 °C for 16 h: anti-Hif1α (1:500, Novus Biologicals, NB100-499), anti-Hmox1 (1:500, Enzo LifeScience, SPA-896F), anti-β-actin (clone C4, 1:5,000, MP Bio, 08691001), or anti-α-tubulin (1:5,000, Sigma-Aldrich, T9026). Next, blots were incubated with secondary antibody at room temperature for 1 h: anti-rabbit or anti-mouse horseradish peroxidase (1:10,000, Agilent Technologies, P0448 (rabbit), P0447 (mouse)). Blots were washed 3 times in TBST after antibody incubations then developed using enhanced chemiluminescent reagent and x-ray film. Densitometry analyses were performed using Image Studio Lite version 5.2.5 (LI-COR Biosciences). For LI-COR imaging, Hif1α blots were incubated at 4 °C for 16 h with anti-Hif1α (clone 54; 1:500, BD Bioscience, 610958). Blots were washed in TBST then incubated in anti-mouse IRDye® 800 (1:5,000, Licor, 925-32210) at room temperature for 1 h, protected from light. Blots were washed in TBST then imaged on the Odyssey Imager (Licor). β-actin (Actb) or α-tubulin (Tuba1A) was used as loading controls in fibroblast experiments. To overcome the lack of a temporally consistent loading control in the hind limb ischemia model, total protein loading determined by Ponceau S staining was used for normalization in these experiments[7].

RNA isolation and qPCR

Skeletal muscle was pulverized in liquid nitrogen, homogenized 2 × 30 s on ice in TRIzol reagent (Thermo Fisher Scientific) then total RNA prepared. cDNA was synthesized from 500 ng total RNA using the SuperScript III First Strand cDNA Synthesis kit and random hexamers as primer, according to the manufacturer's protocol (Thermo Fisher Scientific). Real-time quantitative PCR was performed using the SensiFast SYBR No-Rox kit (Bioline) and a LightCycler 480 (Roche), with primer sets detailed in Supplemental Table 1. *Hmox1* was normalized to the average of three housekeeping genes (*Actb, 18s, Hrpt*) using the ΔΔCT method[8].

Analysis of ATP and AMP content by HPLC-UV

ATP and AMP were detected as described previously[9] using HPLC coupled with UV detection (Agilent 1200 series) with minor modifications. ATP and AMP were separated on a Supelcosil C18 DB column (5 μM, 250 x 4.6 mm) by isocratic elution using 39 mM KH_2PO_4 pH 6.5 at 1 mL/min. ATP and AMP were detected by UV_{254nm} and quantified by area comparison with authentic commercial standards (Sigma Aldrich, USA) and data analysed using ChemStation software (Agilent Technologies, USA).

Metabolite and lipid extraction from muscle tissues

Metabolite and lipid extractions were performed as described previously[10]. 20-55 mg of frozen muscle tissue was weighed and transferred in a lysing matrix tube (6913-500, MP Biomedical) containing 150 μL ice-cold 0.5% (w/v) sodium deoxycholate (SDC, Sigma

Aldrich), cOmplete™ Protease Inhibitor Cocktail (Roche) and internal standard d_8-phenylalanine (Cambridge Isotope Laboratories, USA). The sample was lysed by Precellys 24 homogenizer (Bertin Instrument, France) at the setting of 5,000 rpm, 3 cycles, 30 s cycle, 15 s rest and 4 °C. The lysate was collected and transferred into a fresh centrifuge tube. Protein concentration was estimated by bicinchoninic acid assay. Thereafter, tissue lysate was mixed with 450 µL cold methanol, vortexed and centrifuged at 13,000*g* for 20 min at 4 °C. The supernate (metabolite extract) was collected and evaporated in a speed-vac at ambient temperature. Dried metabolite extracts were resuspended in 100 µL acetonitrile/water (95:5, v/v), centrifuged at 17,000*g* for 10 min at 4 °C and transferred to LC/MS vials for further metabolomics and targeted metabolite analyses.

For lipid extraction, tissue pellets were mixed with 250 µL dichloromethane/methanol (3:1, v/v), 1 µL SPLASH Lipidomix and 100 µL 1 mM butylated hydroxytoluene. Samples were vortexed for 1 min, centrifuged at 17,000*g* for 10 min at 4 °C, transferred to amber LC/MS vials and dried under nitrogen gas. Dried lipid extracts were resuspended in 200 µL chloroform/methanol (1:1, v/v) and subjected to lipidomic LC/MS analysis.

LC/MS analysis of metabolite and lipid extracts

Metabolomic analysis was performed using an Agilent 6560 Q-TOF LC/MS coupled to a 1290 Infinity II UHPLC system. The metabolite extract (10 µL) was subjected to an Agilent 2.7 µm particle 100 x 2.1 mm InfinityLab Poroshell 120 HILIC-Z column maintained at 35 °C and eluted with a gradient using mobile phase A (acetonitrile/water, 95:5, v/v) with 0.1% formic acid and 10 mM ammonium formate and mobile phase B (acetonitrile/water, 1:1, v/v) with 0.1% formic acid and 10 mM ammonium formate. The LC gradient method was as follows: 1% mobile phase B isocratic at 0.4 mL/min for 0-2 min, increase mobile phase B to 55% from 2-8 min at 0.4 mL/min, increase mobile phase B to 99% from 8-9 min at 0.4 mL/min, maintain 99% mobile phase B until 11.1 min at 0.6 mL/min, decrease mobile phase B to 1% from 11.1-14 min at 0.6 mL/min, maintain 1% mobile phase B from 15-20 min at 0.4 mL/min. Metabolites were analysed in positive and negative ion polarity modes. The electrospray settings were as follows: gas temperature 350 °C; drying gas flow 12 L/min; sheath gas temperature 350 °C; sheath gas flow 12 L/min; cap voltage: positive mode 3 kV, negative mode 3.5 kV. Each sample was analysed in MS^1 mode with a scan range of 50-1000 m/z in positive mode, and 50-1100 m/z in negative mode. Triplicates of each sample were pooled and the pooled sample subjected to iterative LC/MS analysis for metabolite identification. Iterative LC/MS method was as follows: each pooled sample was injected 5 times per polarity mode and subjected to LC separation and using the electrospray settings described above. Iterative data acquisition was performed in an Auto MS/MS mode with a scan range of 50-1000 m/z (positive mode) and 50-1100 m/z (negative mode), collision energy 20 eV, 3 precursors per cycle and active exclusion for 0.05 min after 2 spectra. To enable iterative MSMS data acquisition, 'Use PC for MS/MS decision' parameter box must be checked, 'RT exclusion tolerance' should be set at ±0.1 min. Iteration: 'Use PC for MS/MS decision' – tick, 'RT exclusion tolerance' – ± 0.1 min. During each run mass correction was achieved by constant infusion of reference calibration mix ("lock masses") containing molecules producing m/z 121.0509 and 922.0098 ions in positive mode and m/z 112.9856 and 1033.9881 in negative mode.

In addition to the above metabolomic analysis, targeted metabolite analysis was performed on the same tissue metabolite extracts using an Agilent 1290 UHPLC system coupled to an Agilent 6470 triple-quadrupole mass spectrometer. Metabolite extract (4 µL) was subjected to an Agilent Zorbax RRHD Extend-C18 (1.8 µm, 150 x 2.1 mm) with a gradient elution of

metabolites using mobile phase A (3% methanol in water, v/v, containing 10 mM tributylamine and 15 mM acetic acid) and mobile phase B (97% methanol in water, v/v, containing 10 mM tributylamine and 15 mM acetic acid), with the column maintained at 35 °C. The flowrate was set at 0.25 mL/min and gradient program was as follows: increase mobile phase B from 0-20% over 0-7.5 min, increase mobile phase B to 45% from 7.5-13 min, increase mobile phase B to 99% from 13-20 min, maintain 99% mobile phase B from 20-24 min. Thereafter, the column and the guard column were regenerated by a reverse flow with 100% acetonitrile with a flow of 0.25 mL for 3 min. The flow rate was then increased to 0.8 mL/min for 2 min and then reduced to 0.6 mL/min for 45 s. The column was returned to a direct flow and re-equilibrated with mobile phase A at 0.4 mL/min for 8.5 min. Metabolites were analysed in negative electrospray ion mode with capillary voltage set at 2 kV, sheath gas 12 L/min. sheath gas temperature 325 °C, nebulizer pressure 45 psi and scan range 50-1000 m/z.

Lipidomic analysis was performed using an Agilent 6560 Q-TOF LC/MS coupled to a 1290 Infinity II UHPLC system. 5 µL of lipid extract was subjected to a 1.7 µm particle 100 x 2.1 mm ID Waters ACQUITY CSH C18 column maintained at 55 °C and eluted with a gradient using mobile phase A (water/acetonitrile, 4:6, v/v) with 10 mM ammonium formate and mobile phase B (acetonitrile/2-propanol, 1:9, v/v) with 10 mM ammonium formate at a flow rate of 0.3 mL/min. The gradient was as follows: increase in mobile phase B from 0-40% between 0-6 min, increase in mobile phase B from 40-100% from 6-30 min, maintain 100% mobile phase B for another 4 min, and then return to 0% mobile phase B over 2 min where it was kept for 4 min to re-equilibrate the column. Lipids were analysed in positive and negative ionization polarity mode. The electrospray settings were as follows: gas temperature 300 °C; drying gas flow 5 L/min; sheath gas temperature 300 °C; sheath gas flow 12 L/min; cap voltage 3.5 kV. Data acquisition was performed in an Auto MS/MS mode with a scan range of 100-1700 m/z, collision energy 35 eV, 2 precursors per cycle and active exclusion for 0.5 min after 2 spectra. Mass correction during each run was achieved by constant infusion of the reference calibration mix ("lock masses") containing molecules producing m/z 121.0509 and 922.0098 ions in positive mode and m/z 112.9856 m/z and 1033.9881 m/z negative mode.

Analysis of metabolomic and lipidomic data

Analysis of the data from the targeted metabolite analysis was performed using the MassHunter Metabolomics Dynamic MRM Database and Method. Peak shape and area for each metabolite were assessed using the MassHunter Quantitative Analysis software (v. B.04.00). Intensities of identified metabolites were normalised to the intensity of d_8-phenylalanine and protein content. For metabolomics analyses, acquired data were submitted and processed by XCMS online and the METLIN database was used for the identification of shortlisted metabolic features. Intensities of identified metabolites were normalised to the intensity of d_8-phenylalanine and protein content. Acquired lipidomics data files were processed using LipidMatch software[11]. Standards from SPLASH Lipidomix and protein content were used for normalization.

Statistical analysis

Statistical analysis was performed using GraphPrism version 7 software. Results are expressed as mean ± standard error of the mean (SEM). Data was analysed first for normality (D'Agostino & Pearson) and equal variance for continuous variables (excepting % survival curves). If passed, Student's t-test, Kruskal-Wallis one-way ANOVA or two-way ANOVA were performed with pos hoc tests for between group comparisons where appropriate. For

two group comparisons where either test failed the Mann-Whitney rank sum test was used. $P<0.05$ was considered as statistically significant.

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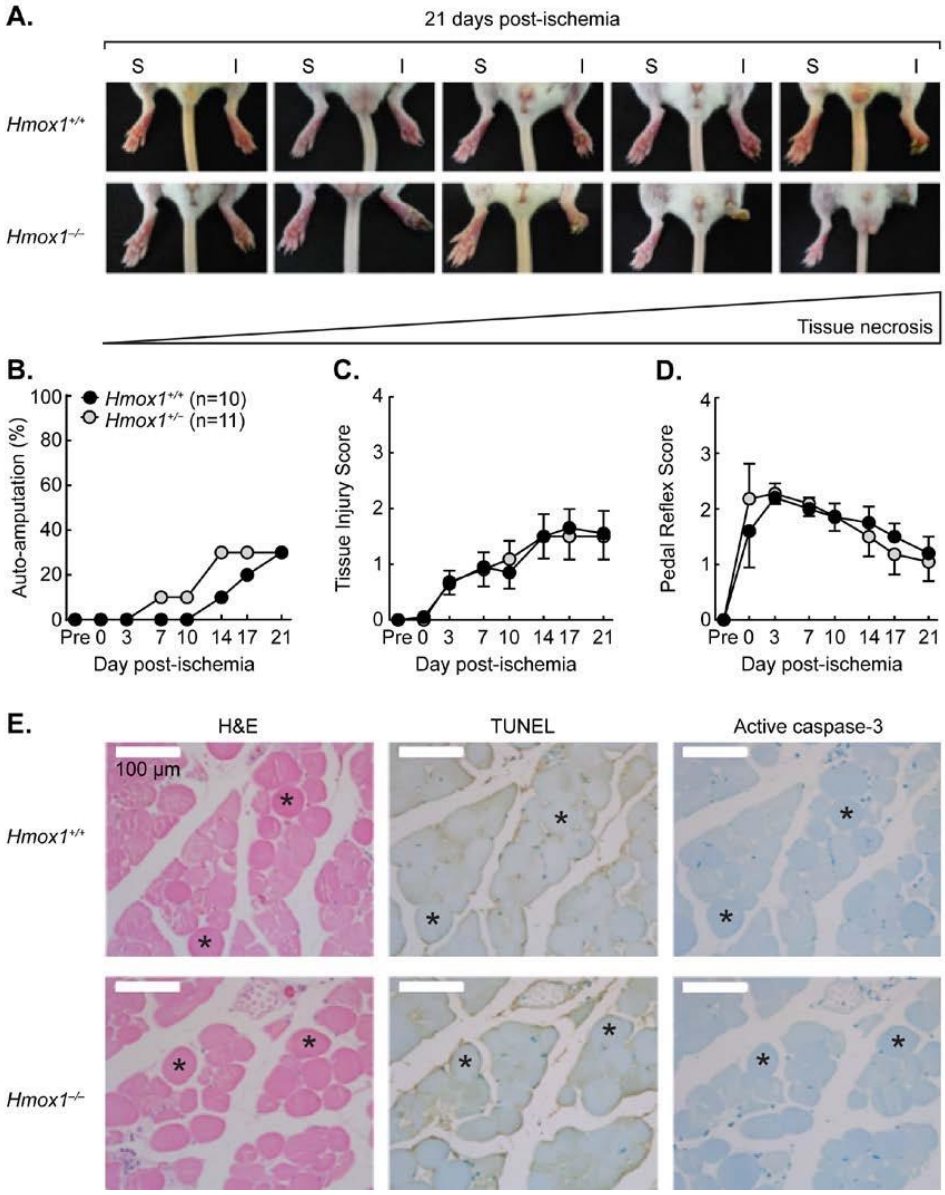
Online Tables and Figures

Online Supplemental Table I. Primers used for qPCR

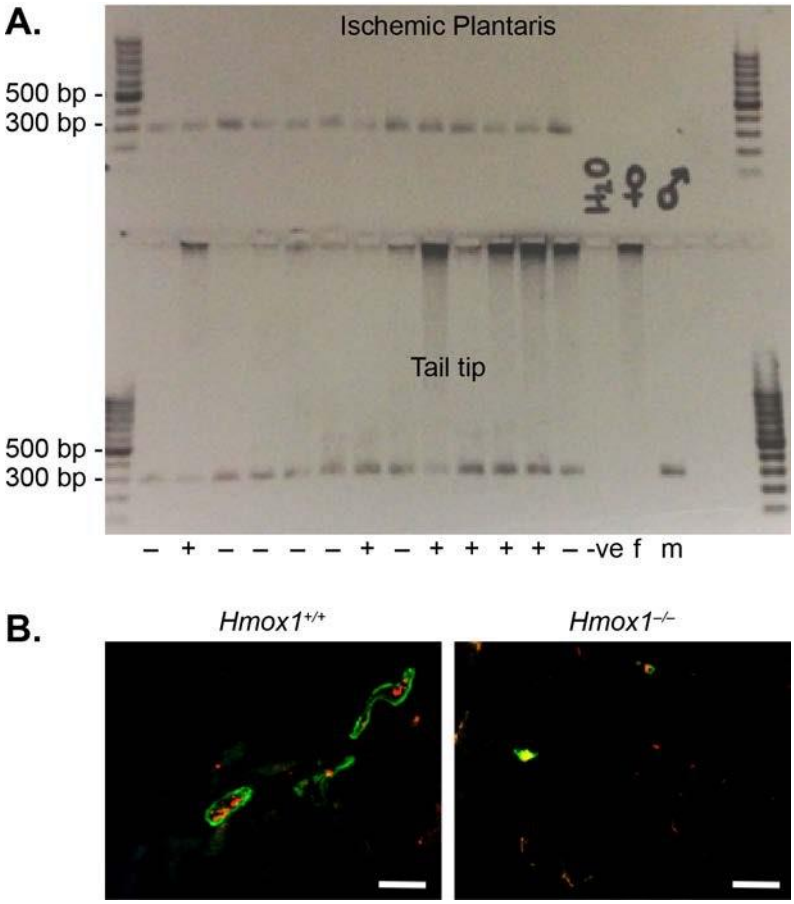
Gene	Forward 5 – 3'	Reverse 5'- 3'
Hmox1	AGGTACACATCCAAGCCGAGA	CATCACCAGCTTAAAGCCTTCT
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Actb	GGATGCAGAAGGAGATCACTG	CGATCCACACGGAGTACTTG
Hprt	GCTTTCCCTGGTTAAGCAGTACA	CAAACTTGTCTGGAATTTCAAATC

Online Supplemental Table II. Skeletal muscle metabolites identified by targeted metabolite analysis as being significantly different in Hmox1$^{-/-}$ mice treated with saline versus DMOG ($p<0.05$)

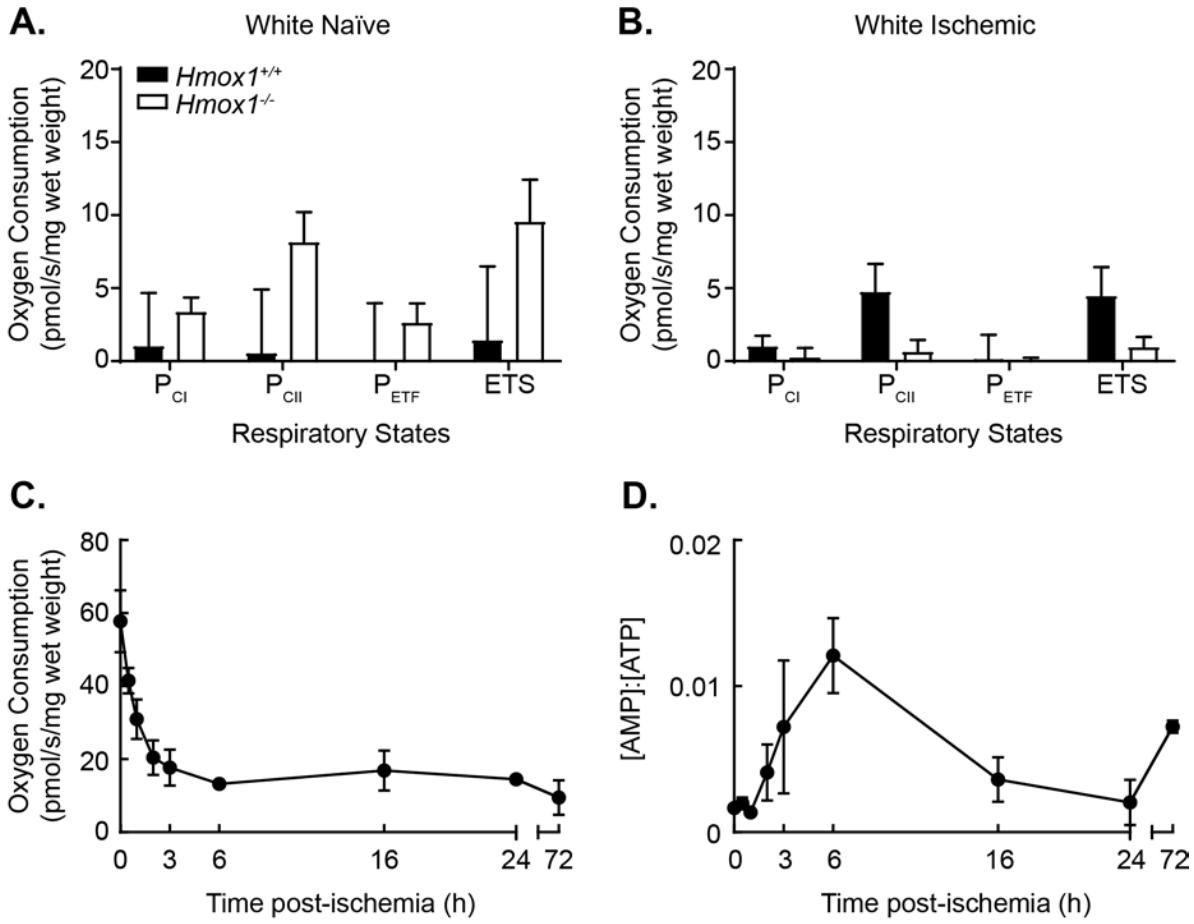
Metabolite	Log2 Fold Change	p-value
Serine	7.73	0.006798
Histidine	6.73	0.040913
Threonine	5.54	0.004511
Tyrosine	2.71	0.011334
Isocitric acid	2.14	6.57E-07
Citric acid	2.13	3.69E-07
Methionine	1.64	0.002447
Proline	1.56	0.042586
Phenylalanine	1.49	0.030624
Succinic acid	1.31	4.11E-05
cis-Aconitic acid	0.91	0.024966
Malic acid	0.78	0.026026
Salicylic acid	0.40	0.049613
Lactic acid	0.35	0.009398
Nicotinamide	-1.04	0.020194
Creatine	-1.05	0.02415
Orotic acid	-1.47	0.008061
5-Deoxy-5-methylthioadenosine	-1.58	0.014471



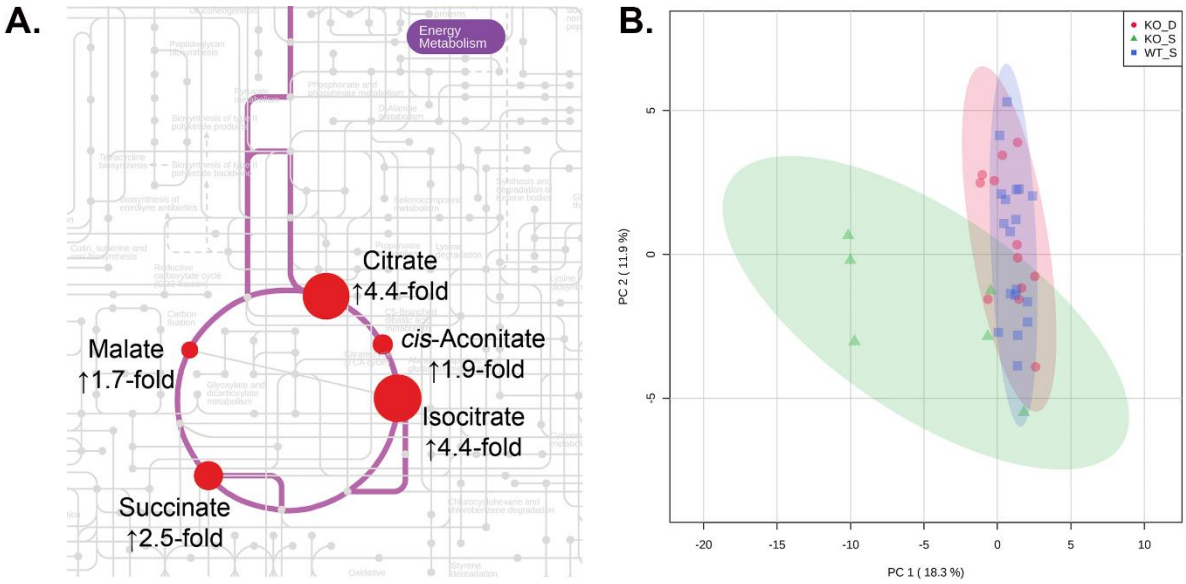
Online Supplemental Figure I.
Unilateral hind limb ischemia was induced in male and female *Hmox1* (n=10), *Hmox1*$^{+/+}$ (n=11) and *Hmox1*$^{+/-}$ mice (n=10), with sham preparation of the contralateral limb. **(A)** Photographs of the sham- operated (S) and ischemic (I) hind limbs demonstrating the range of tissue injury 21 days after ischemia. **(B)** Incidence of auto-amputation. **(C)** Tissue injury score: 0, normal; 1, mild discoloration; 2, necrosis; 3, auto-amputation below the ankle; 4, auto-amputation above the ankle. **(D)** Pedal reflex score: 0, normal; 1, plantar flexion but not toe flexion; 2, no flexion; 3, dragging of the foot; 4, dragging of limb. Data in **(B)-(D)** were enumerated by blinded observers and are expressed as mean ± SEM, n=10 per genotype, and were assessed by 2-way ANOVA with repeated measures in **(C)-(D)** by Sidak's multiple comparison test, *P<0.05. **(E)** Representative photomicrographs of gastrocnemius tissues from naïve mice and mice 24 h after ischemia and stained with hematoxylin and eosin, TUNEL or active caspase- 3 with methylene blue counterstain. Examples of necrotic myocytes (strongly eosinophilic, swollen/oncotic fibers with nuclei loss) are indicated by the asterisk. Scale bar = 100 µm.



Online Supplemental Figure II.
(A) PCR detection of the *sex determining region-Y* gene (at ~300 bp) in ischemic plantaris and tail tip of lethally irradiated female *Hmox1*$^{-/-}$ (-) and *Hmox1*$^{+/+}$ (+) mice reconstituted with male *Hmox1*$^{+/+}$ bone marrow cells. Controls indicated are water only control (-ve), female control (f) and male control (m). **(B)** Representative photomicrographs of vessel density in gastrocnemius tissues from *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ mice; smooth muscle actin positive structures shown in green, CD31 positive structures shown in red. Scale bar = 100 μm.



Online Supplemental Figure III.
Unilateral hind limb ischemia was surgically induced in male and female *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ mice with sham preparation of the contralateral limb as indicated. Electron transfer capacity through complex I (P_{CI}), complex II (P_{CII}), electron transferring flavoprotein (P_{ETF}) and uncoupled oxidative phosphorylation (ETS) in white naïve **(A)** and white ischemic **(B)** muscle fibers of gastrocnemius determined by high-resolution respirometry. **(C)** Maximal oxidative phosphorylation in red gastrocnemius fibers of *Hmox1*$^{+/+}$ mice determined by high-resolution respirometry. **(D)** AMP-to-ATP ratio in mixed gastrocnemius fibers of *Hmox1*$^{+/+}$ mice determined by LC-MS/MS. Data are expressed as mean ± SEM, n=3 mice per time point. Results in **(A)-(B)** were assessed by 2-way ANOVA and in **(C)-(D)** by Kruskal-Wallis test, *P<0.05.



Online Supplemental Figure IV.
Targeted metabolite analysis **(A)** Tricarboxylic acid cycle metabolites (n=5, out of 16) significantly increased in skeletal muscle of *Hmox1*$^{-/-}$ mice treated with saline versus DMOG. **(B)** Principal component analysis (score plot) of skeletal muscle from *Hmox1*$^{-/-}$ mice treated with saline (KO_S) or DMOG (KO_D) and *Hmox1*$^{+/+}$ mice treated with saline based on all metabolites identified by targeted analysis. Extracted feature abundances were normalized to internal standard and tissue protein concentration, transformed (Log transformation and auto-scaling) and checked for normal distribution. Each tissue metabolite extract was analysed by LC/MS as three separate aliquots. Green triangles, *Hmox1*$^{-/-}$ mice treated with saline; red circles, *Hmox1*$^{-/-}$ mice treated with DMOG; blue squares, *Hmox1*$^{+/+}$ mice treated with saline.